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Elucidating the role of Staphylococcus epidermidis serine-aspartate repeat protein G in platelet activation.

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Title Page

Article Title:

Staphylococcus epidermidis serine-aspartate repeat protein G (SdrG) binds platelets by two distinct mechanisms.

Short title:

S. epidermidis protein SdrG activates platelets (47)

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Abstract

Background: S. epidermidis is a commensal of the human skin that has been implicated in infective endocarditis and infections involving implanted medical devices. *S. epidermidis* induces platelet aggregation by an unknown mechanism. The fibrinogen binding protein serine aspartate repeat G (SdrG) is present in 67-91% of clinical strains. *Objectives:* To determine if SdrG plays a role in platelet activation and if so investigate the role of fibrinogen in this mechanism. *Methods*: SdrG was expressed in a surrogate host, *Lactococcus lactis,* in order to investigate its role in the absence of other staphylococcal components. Platelet adhesion and platelet aggregation assays were employed. *Results: L. lactis* expressing SdrG stimulated platelet aggregation (lag time 2.9 ± 0.5 min) whereas the *L. lactis* control did not. *L. lactis* SdrG-induced aggregation was inhibited by $\alpha_{IIb}\beta_3$ antagonists and aspirin. Aggregation was dependent on both fibrinogen and IgG, and the platelet IgG receptor FcγRIIa. Pre-incubation of the bacteria with Bβ-chain fibrinopeptide inhibited aggregation (delaying the lag time 6-fold), suggesting that fibrinogen acts as a bridging molecule. Platelets adhered to *L. lactis* SdrG in the absence of fibrinogen. Adhesion was inhibited by $\alpha_{IIb}\beta_3$ antagonists, suggesting that this direct interaction involves $\alpha_{IIb}\beta_3$. Investigation using purified fragments of SdrG revealed a direct interaction with the B-domains. An indirect mechanism mediated by the A domain involved both a fibrinogen and an IgG bridge. *Conclusion:* SdrG alone is sufficient to support platelet adhesion and aggregation through both direct and indirect mechanisms.

Introduction

Staphylococci are a major cause of intravascular infections that can be difficult to treat due to the high incidence of antibiotic-resistant strains. Staphylococci are divided into coagulase-positive strains such as *Staphylococcus aureus* and coagulase-negative strains such as *Staphylococcus epidermidis. S. epidermidis* is a major cause of hospital acquired infections of indwelling medical devices. Coagulase-negative staphylococci are the leading cause of blood stream infections [1] with *S. epidermidis* implicated in infective endocarditis (IE) [2, [3]. Although contamination of blood products is relatively rare, patients that receive blood or platelet preparations contaminated with *S. epidermidis* can deteriorate rapidly [4]. A significant number of pooled platelet preparations (0.19%) are contaminated with bacteria [5], 67% with *S. epidermidis*.

Both *S. aureus* and *S. epidermidis* have been shown to induce platelet aggregation *in vitro*. *S. aureus* mediates platelet aggregation through multiple mechanisms, many involving fibrinogen binding proteins [6, [7, [8]. The mechanism involved in *S. epidermidis*-induced platelet aggregation is unknown. It was inhibited by treatment of the bacteria with trypsin [9], suggesting that a surface protein is involved, however the protein or proteins involved have not been identified.

The serine aspartate repeat protein SdrG of *S. epidermidis* is a Microbial Surface Component Recognising Adhesive Matrix Molecules (MSCRAMM) that binds fibrinogen. It is also known as Fbe. Deletion of the *sdrG* (*fbe*) gene led to decreased adhesion to fibrinogen-coated surfaces [10], and antibodies to SdrG(Fbe) have been shown to inhibit *S. epidermidis* adhesion on implanted polyethylene catheters [11]. These data suggest that SdrG is the predominant fibrinogen binding protein on *S. epidermidis*. SdrG's closest homologue is the *Staphylococcus aureus* surface protein, SdrE, which can stimulate platelet aggregation in platelet rich plasma (PRP) with a long lag time (13.5 ± 3.5 min) [12]. SdrE does not bind to fibrinogen, and we have

shown the platelet activation mechanism to be complement dependent (Brennan *et al.,* unpublished data). Other homologues on the surface of *S. aureus* involved in platelet activation include the clumping factors ClfA and ClfB and the fibronectin binding proteins FnBPA and FnBPB. These four surface proteins stimulate a faster platelet aggregation dependent on the binding of fibrinogen and IgG [6, [7, [8] (the FnBP's can also use fibronectin instead of fibrinogen).

ClfA and the FnBP's bind to the C-terminus of the γ-chain of fibrinogen [13, [14] while ClfB binds to the C-terminus of the α-chain [15, [16]. SdrG binds to the N-terminus of the Bβ chain (residues 6-20) of fibrinogen [17, [18, [19]. SdrG has the same structural organization as the *S. aureus* Clf-Sdr family of surface proteins [20, [21]. Its extracellular domain is made up of a serine-aspartate repeat region (SD domain), two B-domains and the fibrinogen binding A domain. Ponnuraj *et al.* reported the crystal structure of the ligand binding A domain of SdrG in complex with the fibrinogen Bβ chain N-terminal peptide [21]. The fibrinopeptide was bound in the groove between the N2 and N3 subdomains. The authors demonstrated that the native fibrinopeptide (NEEGFFSARGHRPLD) inhibited SdrG binding to fibrinogen. They also showed that when the underlined serine was replaced by an alanine, the resulting peptide could not inhibit fibrinogen binding. We have used these peptides to investigate the role of fibrinogen in the interaction with platelets.

We postulated that SdrG may mediate *S. epidermidis*-induced platelet aggregation in a manner similar to other fibrinogen-binding MSCRAMMS found on *S. aureus*. In this study, we examined the mechanism of platelet activation stimulated by SdrG. We have demonstrated that two distinct interactions are involved; (i) a direct interaction involving $\alpha_{IIb}\beta_3$ and (ii) an indirect mechanism involving fibrinogen, IgG and the platelet FcγRIIa receptor.

Methods

All compounds were purchased from Sigma Aldrich UK unless stated otherwise.

Platelet Preparation

Blood was collected using a 19-gauge needle from healthy volunteers who had not taken non-steroidal anti-inflammatory drugs during the previous fortnight, without discrimination based on age or sex. Ethical approval was obtained from the RCSI ethics committee. Gel filtered platelets (GFP) and platelet rich plasma (PRP) were prepared as described by Loughman *et al* [7]. The GFPs were diluted to 3 x 10^8 platelets per ml and supplemented with $CaCl_2$ and fibrinogen (Calbiochem, Germany) to final concentrations of 2 μM, and 1 mg/ml, respectively, prior to aggregometry. Platelet counts within the range of 2-4.5 x 10^8 platelets per ml were used in PRP platelet aggregations. Platelet aggregations were carried out using a PAP-4 aggregometer (Biodata Corporation, PA, USA). 100% aggregation represents the light transmission equivalent to the platelet poor plasma control in PRP aggregations, and the buffer control for GFP aggregations. 50μl of bacterial suspension (OD_{600} 1.6) corresponding to 1 x 10^8 bacteria was added to 450μl of PRP or GFPs and incubated at 37°C stirring at 1200 rpm while measuring light transmission. Aggregations were recorded for a maximum of 30 min.

Inhibition Studies

Platelets were incubated with antibodies or inhibitors for 10 min at 37°C prior to the addition of platelet agonists. The inhibitory antibody to GPIIb (CD41), abciximab (Eli Lilly, Indiana, USA), was used at 20 μg/ml. Tirofiban was used at 1 μM, and aspirin at 1 mM (pre-incubation for 5 min). The inhibitory antibody to the FcγRIIa, IV.3 (StemCell Technologies, Vancouver, Canada) was used at 10 μg/ml.

Platelet Adhesion assay

Platelets were prepared by gel filtration as described above. Bacteria were coated onto a 96-well microtiter plate at an OD_{600} of 1.0 for 2 h at 37°C followed by blocking in 1% BSA for 2 h at 37°C. Plates were washed twice in JNL (6 mM dextrose, 130 mM NaCl, 9 mM $NaHCO_3$, 10 mM Na citrate, 10 mM Tris base, 3 mM KCl, 0.8 mM KH_2PO_4 and 0.9 mM $MgCl_2$ pH 7.4), and 50 μl of platelets (2 x 10^8/ml) were added and incubated for 30 min at 37°C. Plates were washed twice in JNL, and lysed in 0.1 M sodium acetate, 0.1% Triton X-100 and 10mM p-nitrophenol phosphate (Pierce, Rockford, IL, USA) for 90 min at 37°C. The plates were read at an absorbance of 405 nm [22].

Depletion of BSA antibodies from pooled human IgG

Gammaguard (Baxter, IL, USA) was depleted of BSA antibodies prior to supplementation into platelet adhesion assays. 6-well plates were coated with 1% BSA in TBS for 2 hours at 37 °C. The plates were washed three times with TBS prior to incubation with 500 μg/ml Gammaguard for 30 min at 37°C. The depleted Gammaguard was removed and stored for use in adhesion assays. Gammaguard IgG and fibrinogen were both used at 50 μg/ml and incubated for 30 min at 37°C prior to platelet adhesions, where indicated.

Bacterial Growth Conditions

Lactococcus lactis MG1363 control and the *L. lactis* expressing SdrE or SdrG were grown as static cultures at 30°C in M17 broth (Oxoid U.K.) supplemented with 0.5% glucose and 5 μg/ml erythromycin.

Staphylococcus epidermidis was grown as static cultures in TSB broth (Difco) at 37°C. *S. epidermidis* overnight starter culture (2 ml) was seeded into 48 ml of TSB broth and incubated at 37°C for 2 hours to grow bacteria to exponential phase. *S. epidermidis* strain 19 was a kind gift from Prof. J.I. Flock, Karolinska Institutet, Huddinge University Hospital, Sweden.

Bacterial Preparation

Bacterial cultures were centrifuged at 5,000 x g for 10 min. The broth was discarded, the pellets washed in PBS, and centrifuged again at 5,000 x g for a

further 10 min. The resulting pellets were resuspended in PBS to the relevant optical density (OD), measured using the Ultrospec III spectrophotometer (Pharmacia Biotech U.K.), at 600nm. The optical density, (OD_{600}) of 1.6 corresponds to 7 x 10^9 colony forming units (CFU), and OD_{600} of 1.0 corresponds to 3 x 10^9 CFU.

Bacterial Adhesion Assays

Adhesion assays were carried out according to Hartford *et al* [23]. Briefly, plates were coated with fibrinogen (Calbiochem, Darmstadt, Germany) for 2 h at 37°C or for 16 h at 4°C. Plates were blocked with 1% BSA at 37°C for 1 h, and washed 3 times in PBS. Bacteria were incubated at 37°C for 2 h at an OD_{600} of 1. Adherent bacteria were washed three times in PBS, and fixed in 25% formaldehyde at room temperature for 30 min. Wells were washed three times in PBS, and stained using 5% crystal violet for 10 min. Wells were washed three times in PBS, and the crystal violet solubilized using 5% acetic acid. Plates were read at 620 nm using a Wallac Victor^2, (Shelton, Connecticut, USA).

Peptide Synthesis

Peptides were prepared by Solid Phase Peptide Synthesis [24]. Peptide masses were confirmed by Matrix Assisted Laser Desorption Ionisation – Time Of Flight (MALDI-TOF) and peptide purity was determined to be greater than 95% for all experiments.

Synthesis and Purification of Recombinant SdrG Fragments

The putative ligand binding A domain of SdrG and the B1-B2 domain was expressed in *Escherichia coli* TOPP3 with a hexahistidine tag at the N-terminus [12]. Recombinant proteins were purified using Ni^{2+} chelate chromatography [25].

Statistics

Statistics were carried out using InStat version 3 (Graphpad Software Inc.). Experiments were carried out 3-5 times and data analysed by ordinary ANOVA followed by the Dunnett's test unless otherwise stated. When there were only two data sets to be compared, a paired two-tailed student's T-test

was carried out. *P* values less than 0.05 were considered significant and have been illustrated with a single star. Values less than 0.01 were considered highly significant, illustrated with a double star. Repeated measures ANOVA was carried out where the data was paired.

Results

Platelet Aggregation in PRP

The wild type *S. epidermidis* strain 19 is known to express SdrG in the exponential phase of growth [26]. These bacteria stimulated platelet aggregation (67 ± 10%; n=3), with a lag time of 7.8 ± 3.5 min (time taken from the addition of bacteria to the beginning of the aggregation response; Table 1). When SdrG was over-expressed on the surrogate host *L. lactis*, platelets were activated with a shorter lag time of 2.9 ± 0.5 min (73 ± 9%). These aggregations were completely inhibited (no aggregation within 30 min) by the $\alpha_{IIb}\beta_3$ antagonists abciximab and tirofiban, and also by aspirin, (0.3 ± 0.3%, 0.7 ± 0.7% and 16 ± 6% respectively data not shown). These data suggest that this was true aggregation and not agglutination. The host bacteria *L. lactis*, which does not express SdrG, did not support aggregation and was used as a control (no aggregation after 30 min, 0.3 ± 0.3%).

Role of Fibrinogen Binding in SdrG-induced Platelet Aggregation

In order to investigate the role of fibrinogen in SdrG-induced platelet aggregation, we used the N-terminal peptide derived from Bβ chain fibrinogen peptide, NEEGFF**SAR**GHRPLD, (SAR), which has been shown to inhibit binding of recombinant SdrG to fibrinogen [21]. The authors demonstrated that when the serine was replaced with an alanine, NEEGFF**AAR**GHRPLD, (AAR), the variant peptide did not inhibit the SdrG-fibrinogen interaction. We thus used this peptide as a control. The peptides that we synthesised for this study displayed the same activity. The SAR peptide (100 μM) inhibited adhesion of *L. lactis* SdrG to immobilized fibrinogen by 81.6 ± 3.4% ($P < 0.05$) while the control AAR peptide had no significant effect; 8.0 ± 4.2% inhibition (data not shown).

Incubation of *L. lactis* expressing SdrG with the native SAR peptide (5 mM) delayed the lag time to platelet aggregation from 1.6 ± 0.4 min to 8.9 ± 1.7 min ($P < 0.05$; figure 1A). The control AAR peptide had no effect on platelet

aggregation (with 5 mM the lag time was 1.1 ± 0.5 min). The SAR peptide reached a maximum effect at 5 mM (figure 1B) whereas no significant effect was seen with the control AAR peptide even at 10 mM (lag time 2.7 ± 1.2 min). These peptides had no significant effect on platelet aggregation stimulated by *L. lactis* SdrE (lag times 10.9 ± 1.8 min, SAR-treated 9.8 ± 3.5 min, AAR-treated 10.5 ± 4.0 min, figure 1A). These peptides also had no significant effect on aggregation promoted by *S. aureus* Newman (lag times 0.5 ± 0.1 min, SAR-treated 0.5 ± 0.1 min, AAR-treated 0.5 ± 0.1 min, figure 1A). Figure 1C is a representative aggregation trace demonstrating the effect of the peptides on platelet aggregation induced by *L. lactis* SdrG. These peptides also had no effect on arachidonic acid induced platelet aggregation.

Role of IgG in SdrG Mediated Platelet Aggregation

L. lactis SdrG failed to induce aggregation of washed platelets alone or supplemented with fibrinogen (figure 2A). However, the addition of both fibrinogen and IgG to washed platelets was sufficient to support platelet aggregation. Furthermore pre-coating the bacteria with IgG and supplementing with soluble fibrinogen also supported aggregation, demonstrating that the IgG was bound to the surface of the bacteria during aggregation. The percentage aggregation and lag times taken to aggregation is summarized in figure 2C. A representative platelet aggregation trace depicting aggregation stimulated by *L. lactis* SdrG when supplemented with fibrinogen and IgG is shown in figure 2D. The *L. lactis* control did not stimulate aggregation under these conditions. The dependence on IgG led us to investigate the role of the platelet IgG receptor, FcγRIIa. *L. lactis* SdrG-induced aggregation was inhibited by a monoclonal antibody directed against the FcγRIIa, IV.3, figure 2B; *L. lactis* SdrG aggregation 58.0 ± 5.7%, IV.3 treated 2.3 ± 0.3%, $P < 0.01$; *L. lactis* SdrG lag time 2.3 ± 1.1 min, IV.3 treated did not aggregate after 30 min).

Platelet Adhesion to SdrG

Immobilized *S. epidermidis* 19 supported platelet adhesion of washed platelets (106 ± 4.5% of the immobilized fibrinogen control). *L. lactis* SdrG also supported platelet adhesion (209 ± 65%) while the *L. lactis* control failed

to support platelet adhesion (7.9 ± 3.0%); figure 3). Adhesion of washed platelets to immobilized *L. lactis* SdrG was inhibited by the $\alpha_{IIb}\beta_3$ antagonists, abciximab (24.9 ± 16.4%, $P < 0.05$) and tirofiban (35.8 ±14.6%, $P < 0.05$), suggesting a direct interaction between SdrG and $\alpha_{IIb}\beta_3$. Subsequently, we purified the recombinant A and B domains of SdrG (figure 4) in order to further localise the platelet binding region of SdrG. Platelet adhesion to the immobilized A domain was weak (18 ± 7% of the fibrinogen control). This adhesion was enhanced by the addition of fibrinogen or IgG alone, (30 ± 8%, 41 ± 10% respectively) and together 52 ± 6% ($P < 0.01$ for all). The immobilized B domain supported platelet adhesion at 33 ± 4% of the fibrinogen control. This adhesion was not significantly enhanced by the presence of fibrinogen or IgG, added prior to the platelets (figure 5A, fibrinogen only, 34 ± 6%; IgG only, 43 ± 9%; fibrinogen and IgG, 39 ± 7%). The $\alpha_{IIb}\beta_3$ antagonist tirofiban inhibited adhesion to the B-domain by 78.3% ($P < 0.05$) suggesting that RGD binding site on the integrin is involved (figure 5B). These purified proteins did not stimulate aggregation in platelet rich plasma (PBS control 2.7 ± 0.3%, A-domain 3.7 ± 0.9%, B-domain 5.3 ± 0.9%, percentages measured at 10 min, n=3, data not shown).

Discussion

SdrG is present in 67-91% of clinical strains [27]. A recent study demonstrated that SdrG expression was induced in a strain that did not express detectable levels of SdrG *in vitro,* after injection into the blood stream of mice [28]. Furthermore, McCrea *et al.* have shown that patients recovering from *S. epidermidis* infections have an increased antibody titer for SdrG when compared to age matched controls [29]. This suggests that SdrG is expressed on the surface of *S. epidermidis* during infection, and is thus clinically important.

Wild type *S. epidermidis* strain 19 induced platelet aggregation which is likely due to the presence of SdrG that is known to be expressed on its surface [26]. *L. lactis* SdrG induced platelet aggregation, although with a shorter lag time. This difference in lag time is probably due to the increased levels of SdrG in *L. lactis* SdrG. We have previously shown that the level of expression of a pro-aggregatory surface protein is an important determinant of lag time [7]. Platelet aggregation was inhibited by $\alpha_{IIb}\beta_3$ antagonists and aspirin suggesting that SdrG stimulates true aggregation via $\alpha_{IIb}\beta_3$ that is dependent on cyclooxygenase signaling.

The N-terminal peptide of the Bβ chain of fibrinogen NEEGFFSARGHRPLD has been shown to inhibit fibrinogen binding to SdrG. Addition of this peptide allowed us to study the effects of SdrG on platelet function in the absence of fibrinogen binding to the bacterial surface while maintaining fibrinogen levels necessary to support platelet aggregation. Addition of the SAR peptide prolonged the lag time of *L. lactis* SdrG-induced platelet aggregation 6-fold to 8.9 ± 1.7 min. SdrE is a homologous protein to SdrG that is found on *S. aureus* which induced platelet aggregation with a similarly long lag time of 10.9 ± 1.8 min this study, (13.5 ± 3.5 min [12]). Thus inhibition of the SdrG fibrinogen mechanism delays the lag time to *L. lactis* SdrG aggregation to a time similar to that taken to induce *L. lactis* SdrE aggregation. This result confirms that fibrinogen bound to the bacterial cell surface is required for rapid

platelet aggregation to take place. This is unlikely to be due to inadequate levels of the SAR peptide as the concentration used completely inhibited fibrinogen binding and increasing the concentration further did not prolong the lag time further.

The fact that inhibition of fibrinogen binding could only prolong the lag time and not prevent platelet aggregation suggests that both SdrE and SdrG have a fibrinogen-independent mechanism (probably complement-dependent) for platelet activation as was found for the *S. aureus* MSCRAMMs ClfA and ClfB [7, [8]. Bacteria expressing a ClfA or ClfB mutant which could not bind fibrinogen had a delayed lag time to aggregation and required complement fixation to occur on the cell surface [7, [8].

The presence of fibrinogen alone was insufficient to support platelet aggregation because with washed platelets both fibrinogen and IgG were required (figure 2). This is analogous to the mechanism demonstrated for *S. sanguis* M99, *S. salivarius* D1 [30], the *S. aureus* surface proteins, ClfA [7], ClfB [8] and FnBPA [6], and *S. pyrogenes* M protein [31]. For all these bacteria, both fibrinogen and IgG are required for aggregation. In the case of FnBPA, fibronectin and IgG can also support aggregation, while IgG alone cannot [6]. In the case of *H. pylori*, aggregation is dependent on bridging by both vWf with GPIb and IgG with FcγRIIa [32]. Collectively it appears that antibody binding to a surface antigenic protein alone is insufficient to stimulate platelet aggregation, but that a second bridging mechanism is required. In the case of SdrG, it has been demonstrated that patients recovering from *S. epidermidis* infections have an increased antibody titer for SdrG [29], and therefore specific antibody is present in patients. We have demonstrated a role for IgG in platelet aggregation and that its second bridging protein is fibrinogen. This mechanism is analogous to that of ClfA. However ClfA binds to a different domain of fibrinogen. Although ClfA, ClfB and SdrG bind to different regions on fibrinogen, they can all support aggregation. This is not unreasonable as the fibrinogen molecule is large, and the areas which SdrG, ClfA, FnBPA or ClfB bind to do not block the RGD sites on fibrinogen which

are recognized by $\alpha_{IIb}\beta_3$. In contrast, *L. lactis* SdrE-induced platelet aggregation is dependent on a plasma factor other than fibrinogen [12] and thus cannot stimulate aggregation of washed platelets, even when supplemented with fibrinogen and IgG. This further confirms that fibrinogen bound to the bacterial cell is involved in the initial interaction of SdrG with platelets in order to promote fast aggregation.

Unlike other Clf-Sdr proteins, *L. lactis* SdrG is able to support platelet adhesion directly without any plasma protein acting as a bridge. This suggests a direct interaction with a platelet receptor. Platelet adhesion to *L. lactis* SdrG was inhibited by tirofiban suggesting an interaction with $\alpha_{IIb}\beta_3$. There are a number of RGD-like sequences in the SdrG sequence, (79-KND-81, 404-KLD-406, 544-KYD-546 and 549-KDD-551 in the A domain and 643-RTD-645 in the B domain), thus we purified the extracellular A and B domains for further experiments. Purified A and B domain fragments of SdrG both supported platelet adhesion. The A domain binds fibrinogen and it was therefore not surprising that the weak adhesion was enhanced in the presence of fibrinogen. The A domain also supported enhanced adhesion in the presence of pooled human IgG. We therefore suggest that there are two independent interactions between the A domain and platelets; a fibrinogen-$\alpha_{IIb}\beta_3$ interaction, and an IgG-FcγRIIa interaction. We propose that this mechanism can be extrapolated to aggregation where both of these interactions are required for platelet signalling to take place. It is possible that the direct interaction is also involved in platelet aggregation, however this alone was insufficient to stimulate platelet aggregation because both fibrinogen and IgG were required to activate washed platelets (figure 2). The B-domain had a much stronger direct interaction with platelets that was not influenced by fibrinogen binding. Adhesion to the B-domain was inhibited by the $\alpha_{IIb}\beta_3$ antagonist tirofiban. The B domain contains an RGD-like sequence RTD (figure 4). The RGD sequence is the sequence responsible for the interaction between fibrinogen and the platelet receptor $\alpha_{IIb}\beta_3$. Therefore the RTD sequence in the SdrG B-domain might be responsible for the interaction between the SdrG B domains and platelets. We cannot rule out that there

may be residual levels of fibrinogen in our platelet preparation, or that the platelets may secrete fibrinogen during the adhesion assay, which could be involved in the platelet adhesion assay. The fact that the direct adhesion seems to be through the B-domain suggests that this is not the case, as the B-domain does not interact with fibrinogen.

In summary, the *S. epidermidis* surface protein SdrG can stimulate platelet activation and supports platelet adhesion. It achieves this through two distinct mechanisms, one involving a direct interaction between the B domain and the platelet integrin $\alpha_{IIb}\beta_3$, and a second using fibrinogen and IgG as bridging molecules to the integrin and the FcγRIIa receptor respectively (figure 6). In infective endocarditis the direct interaction between SdrG and $\alpha_{IIb}\beta_3$ may act to support platelet adhesion to infected valves while the fibrinogen mediated interaction with $\alpha_{IIb}\beta_3$ leads to platelet activation and subsequent thrombus formation. However, as the K_d of SdrG is in the range of 90-300 nM [17, [21] and plasma fibrinogen levels are around 10-fold in excess of this, it is likely that the fibrinogen/antibody interaction is the dominant interaction *in vivo.* The binding sequence leading to platelet aggregation cannot be demonstrated, however platelet signalling is required for activation. Outside-in signalling could take place through either the direct interaction between the B-domain and $\alpha_{IIb}\beta_3$, or through antibody binding to the FcγRIIa. A further possibility is that the binding of fibrinogen to SdrG leads to conformational changes in the fibrinogen molecule, similar to changes seen when fibrinogen is immobilized on a solid surface, allowing it to interact with resting $\alpha_{IIb}\beta_3$. Once this initial outside-in signal takes place, inside-out signalling leads to the activation of $\alpha_{IIb}\beta_3$ allowing it to interact with soluble fibrinogen leading to crosslinking of platelets, resulting in aggregation. As no single initial activation stimulus alone was sufficient to stimulate inside-out signalling and thus aggregation, it is not possible to suggest which binding takes place first. A recent paper by Newman and co-workers demonstrated that the FcγRIIa is required for 'outside-in' signaling events stimulated by platelets binding to fibrinogen [33]. Therefore it is possible that both interactions must take place for platelet activation.

Blocking the interaction between SdrG and platelets may abrogate the thrombotic complications associated with *S. epidermidis* infections. *S. epidermidis* strains expressing high levels of SdrG should be considered more pathogenic than other strains as they not only have an increased capacity to colonize indwelling devices conditioned with deposited fibrinogen, but also to support platelet adhesion and activation. Thus SdrG-expressing strains are likely to lead to platelet build up on indwelling devices, and possibly to further thrombotic complications.

Acknowledgments

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References

Figure Legends

Figure 1: Inhibition of platelet aggregation by the fibrinogen Bβ chain C-terminal peptide. (A) *L. lactis* SdrG or *L. lactis* SdrE was incubated with the native fibrinopeptide (NEEGFF**SAR**GHRPLD), or the control peptide (NEEGFF**AAR**GHRPLD) for 10 min prior to platelet aggregation in platelet rich plasma (a single star represents $P < 0.05$, n > 3). (B) Dose response curve of the SAR peptide for *L. lactis* SdrG-induced aggregation (n > 3). (C) Representative aggregation trace demonstrating the effect of the SAR peptide at 5 and 10 mM, and the control AAR peptide at 10 mM.

Figure 2: SdrG-induced platelet aggregation is dependent on fibrinogen, IgG and the FcγRIIa receptor. (A) Aggregations were carried out in gel filtered platelets supplemented with 2 mM $CaCl_2$. Fibrinogen (1 mg/ml) and IgG (500 μg/ml) were supplemented back into the aggregations either individually or combined. *L. lactis* SdrG and *L. lactis* control were coated with IgG for 30 min at 37 °C and washed in TBS prior to aggregation (indicated as coat). The fibrinogen in this experiment was depleted of contaminating IgG using a Sepharose protein A column (percentage aggregation was measured 10 min after the start of the experiment). (B) Aggregations were carried out in PRP. Platelets were incubated with the FcγRIIa monoclonal antibody (IV.3) or TBS for 10 min at 37°C prior to aggregations (Percentages were measured 5 min after the *L. lactis* SdrG (control) started to aggregate, $P < 0.05$, n=3). (C) Percentage aggregation and lag times relating to (A). No aggregation represents a lack of aggregation response at the termination of the experiment at 15 min. (D) Representative platelet aggregation trace of *L. lactis* SdrG aggregation supplementation assay.

Figure 3: *L. lactis* SdrG platelet adhesion is inhibited by $\alpha_{IIb}\beta_3$ antagonists. *L. lactis* SdrG, *L. lactis* control or fibrinogen (20 μg/ml) were coated onto the 96-well plate and blocked with 1% BSA prior to platelet adhesion with GFPs supplemented with fibrinogen. Tirofiban and abciximab were incubated with the platelets for 10 min prior to the adhesion (n=3).

Repeated measures ANOVA was carried out followed by the Dunnett's post-test using the *L. lactis* SdrG adhesion as the control comparator. A single star denotes ($P < 0.05$), and a double star $P < 0.01$.

Figure 4: SdrG sequence and structure. The overall structure of SdrG is outlined in the schematic in (A). (B) Represents the sequence of SdrG with important domains and sequences identified. The signal sequence, S, (residues 1-50) is followed by the fibrinogen binding A domain (residues 51-618). The A domain is followed by the B domain which has been underlined, and the RGD-like sequence (RTD) is highlighted in bold. The serine-aspartate (SD) repeat region is highlighted in grey. The C-terminal cell wall anchoring LPXTG motif is underlined and highlighted in bold. The hydrophobic membrane-spanning domain is underlined.

Figure 5: Platelets adhere directly to the B domain of SdrG. Purified proteins were coated onto microtiter plates at 50 μg/ml prior to platelet adhesion. The plates were blocked with 1% BSA, washed with TBS and then incubated with either TBS, fibrinogen (50 μg/ml), IgG (50 μg/ml), or IgG (50 μg/ml) and fibrinogen (50 μg/ml) together, for 30 min at 37ºC. The plates were then washed and incubated with gel-filtered platelets (supplemented with 2 mM $CaCl_2$) for 30 min 37ºC. Data was normalized to the fibrinogen control. One-way ANOVA was carried out followed by the Dunnett's post test, using the TBS control as the comparator (double stars represent $P < 0.01$, n=3).

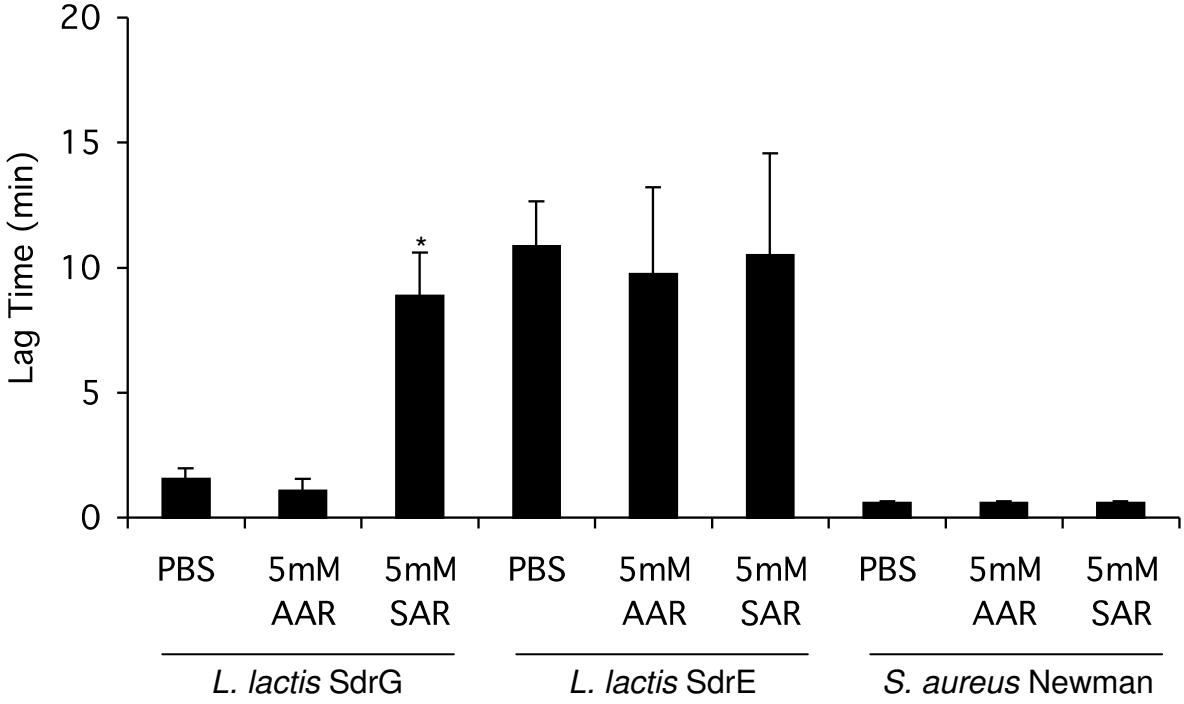
Figure 6: Schematic diagram of the interaction between *S. epidermidis* surface protein SdrG and platelets. The A domain of SdrG interacts with the platelet in a fibrinogen and IgG dependent manner, while the B domain interacts directly with $\alpha_{IIb}\beta_3$, likely through the RTD sequence in the B1 domain. This direct interaction is evident during platelet adhesion, although it cannot support platelet aggregation.

Tables

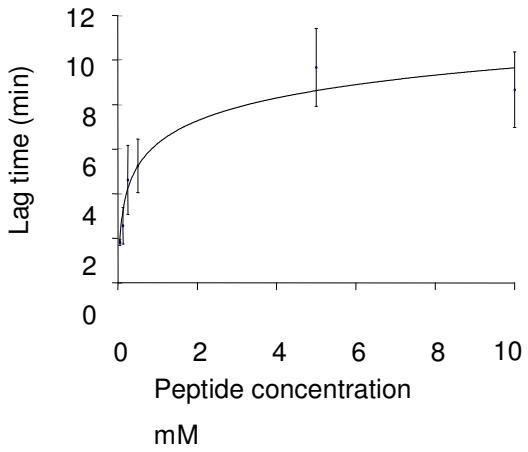
Table 1: *L. lactis* SdrG platelet aggregation is inhibited by $\alpha_{IIb}\beta_3$ antagonists and aspirin. No aggregation implies that there was no aggregation stimulated at 30 min. Percentage aggregation represents the percent aggregation at 30 min.

	Lag Time (min)	**Percentage Aggregation**
***L. lactis* control**	no aggregation	0 ± 0%
***L. lactis* SdrG**	2.9 ± 0.5	73 ± 9% (n=5)
+ Abciximab	no aggregation	0 ± 0% ($P < 0.01$, n=4)
+ Tirofiban	no aggregation	1 ± 1% ($P < 0.01$, n=3)
+ Aspirin	no aggregation	16 ± 6% ($P < 0.01$, n=4)
***S. epidermidis* 19**	7.8 ± 3.5	67 ± 10% (n=3)
+ Tirofiban	no aggregation	0 ± 0% ($P < 0.01$, n=3)

A


20
15
10
5
0
Lag Time (min)
*
PBS
5mM AAR
5mM SAR
PBS
5mM AAR
5mM SAR
PBS
5mM AAR
5mM SAR
L. lactis SdrG
L. lactis SdrE
S. aureus Newman

B


12
10
8
6
4
2
0
Lag time (min)
0
2
4
6
8
10
Peptide concentration
mM

C

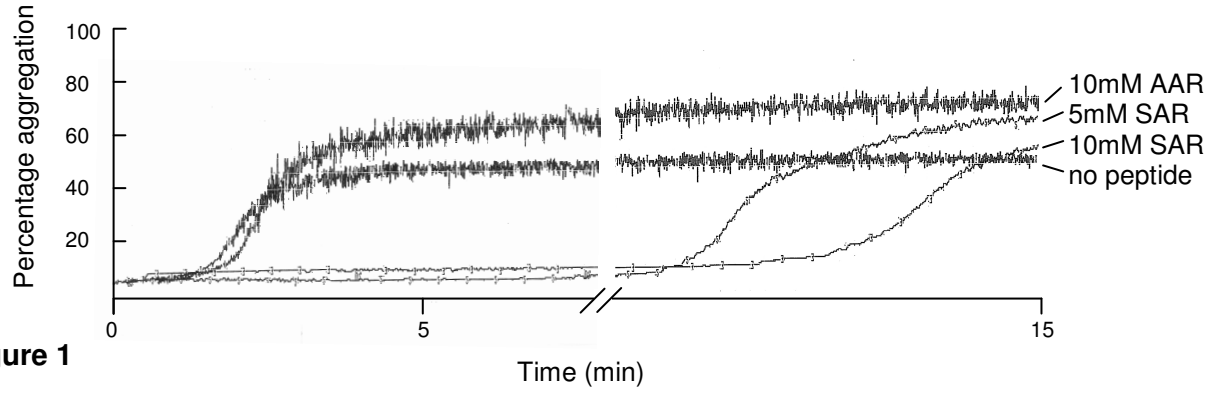

100
80
60
40
20
Percentage aggregation
0
5
15
Time (min)
10mM AAR
5mM SAR
10mM SAR
no peptide

Figure 1

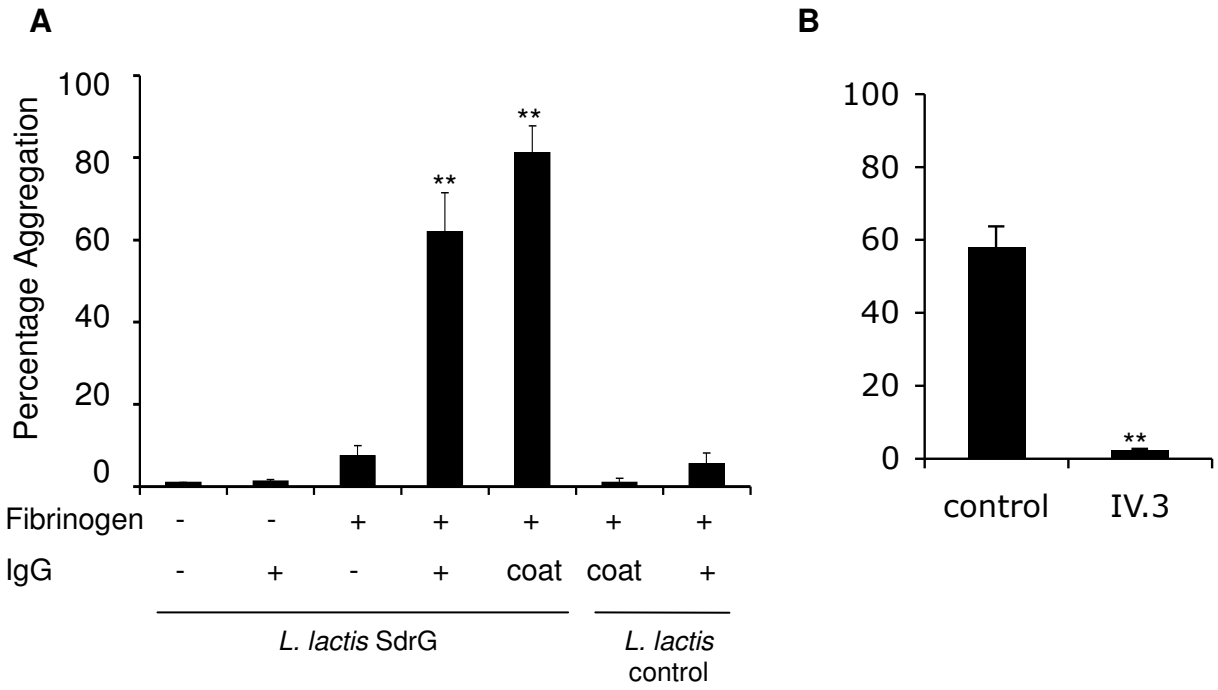
Figure 1D

Percentage Inhibition by peptides

	AAR (control)	**SAR**	
***L. lactis* SdrG**	13 ± 8% (*NS)*	83 ± 11% (*P* < 0.01)	N=5
***L. lactis* SdrE**	0 ± 0% (*NS)*	0 ± 0% (*NS)*	N=3
***S. aureus* Newman**	5 ± 2% (*NS)*	3 ± 2% (*NS)*	N=4
Arachidonic acid	0 ± 0% (*NS*)	5 ± 5% (*NS*)	N=4
TRAP	2 ± 1% (*NS*)	4 ± 3% (*NS*)	N=3

Percentage Aggregation (5 min after the control starts to aggregate)

	PBS	**AAR**	**SAR**
***L. lactis* SdrG**	46 ± 4% (n=5)	45 ± 6% (*NS*, n=5)	8 ± 5% (*P* < 0.01, n=5)
***L. lactis* SdrE**	41 ± 6% (n=5)	43 ± 6% (*NS*, n=3)	42 ± 3% (*NS,* n=3)
***S. aureus* Newman**	72 ± 5% (n=4)	67 ± 6% (*NS,* n=4)	69 ± 4% (*NS,* n=4)
Arachidonic Acid	76 ± 6% (n=4)	85 ± 5% (*NS,* n=4)	92 ± 3% (*NS*, n=4)
TRAP	93 ±2% (n=3)	90 ± 1% (NS, n=3)	92 ± 0% (NS, n=3)



C

	Fibrinogen	IgG	Lag time to aggregation	Percentage aggregation	
L. lactis SdrG	-	-	no aggregation	1.0 ± 0.0%	N=3
	-	+	no aggregation	1.0 ± 0.3%	N=3
	+	-	no aggregation	7.5 ± 2.5%	N=4
	+	+	4.3 ± 0.9 min	62.0 ± 9.5% (P<0.01)	N=4
	+	coated	1.7 ± 0.1 min	81.3 ± 6.5% (P<0.01)	N=4
L. lactis control	+	coated	no aggregation	1.0 ± 1.0%	N=4
	+	+	no aggregation	5.5 ± 2.7%	N=4

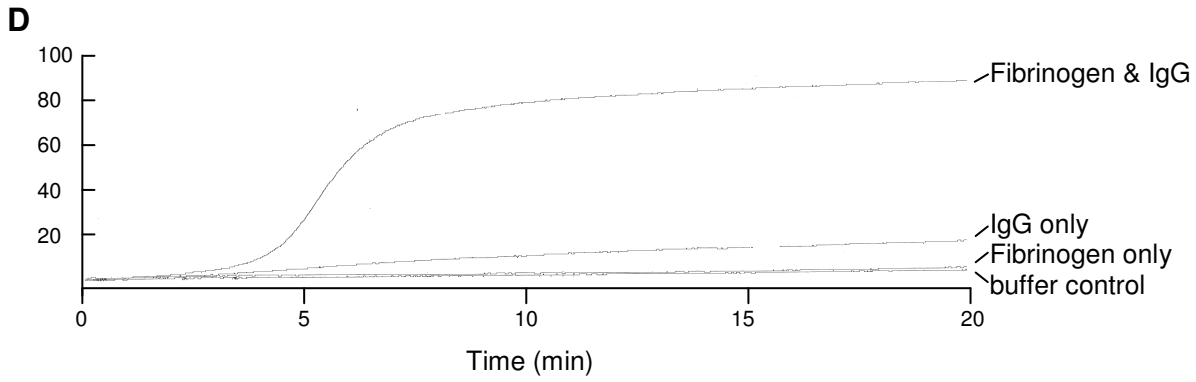


Figure 2

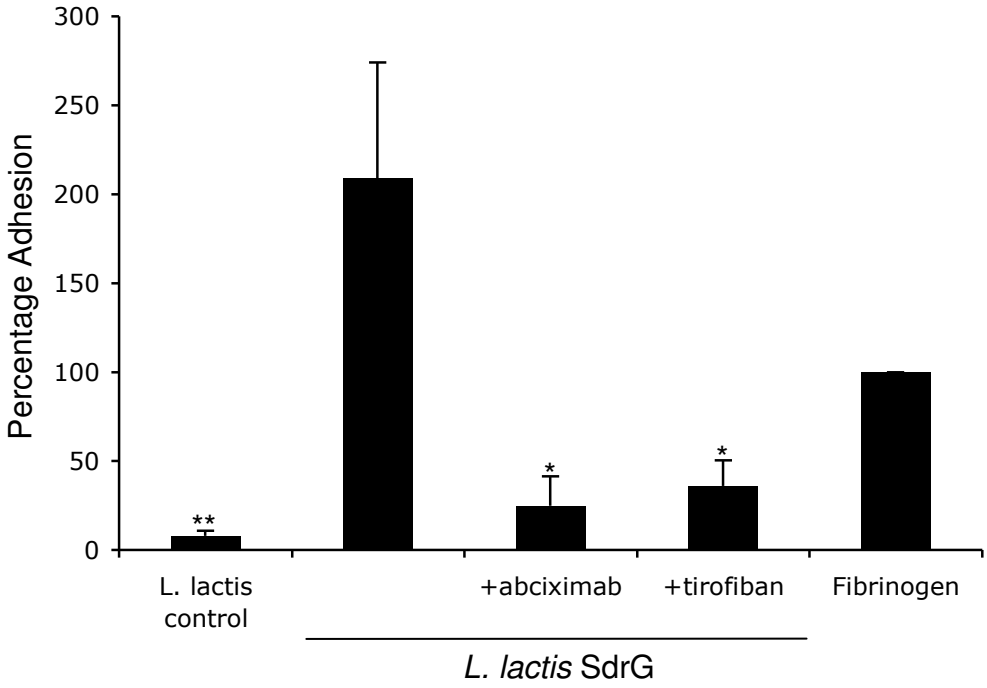
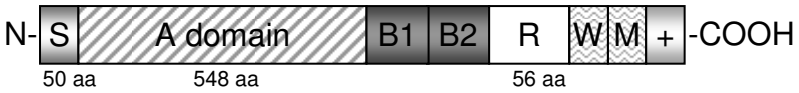

300
250
200
150
100
50
0
Percentage Adhesion
**
*
*
L. lactis control
+abciximab
+tirofiban
Fibrinogen
L. lactis SdrG

Figure 3

A



B

Signal sequence (S) ***A domain***

```
1   MIKKNNLLTKKKPIANKSNKYAIRKFTVGTASIVIGAALLFGLGHNEAK AEENTVQDVKD  60

61  SNMDDELSDSNDQSSNEEKNDVINNSQSINTDDDNQIKKEETNSNDAIENRSKDITQSTT 120

121 NVDENEATFLQKTPQDNTQLKEEVVKEPSSVESSNSSMDTAQQPSHTTINSEASIQTSDN 180

181 EENSRVSDFANSKIIESNTESNKEENTIEQPNKVREDSITSQPSSYKNIDEKISNQDELL 240

241 NLPINEYENKVRPLSTTSAQPSSKRVTVNQLAAEQGSNVNHLIKVTDQSITEGYDDSDGI 300

301 IKAHDAENLIYDVTFEVDDKVKSGDTMTVNIDKNTVPSDLTDSFAIPKIKDNSGEIIATG 360

361 TYDNTNKQITYTFTDYVDKYENIKAHLKLTSYIDKSKVPNNNTKLDVEYKTALSSVNKTI 420

421 TVEYQKPNENRTANLQSMFTNIDTKNHTVEQTIYINPLRYSAKETNVNISGNGDEGSTII 480

481 DDSTIIKVYKVGDNQNLPDSNRIYDYSEYEDVTNDDYAQLGNNNDVNINFGNIDSPYIIK 540

541 VISKYDPNKDDYTTIQQTVTMQTTINEYTGEFRTASYDNTIAFSTSSGQGQGDLPPEKTY 600
                    B domains
601 KIGDYVWEDVDKDGIQNTNDNEKPLSNVLVTLTYPDGTSKSV RTDEEGKYQFDGLKNGLT 660

661 YKITFETPEGYTPTLKHSGTNPALDSEGNSVWVTINGQDDMTIDSGFYQTPKYSLGNYVW  720
                B2
721 YDTNKDGIQGDDEKGISGVKVTLKDENGNIISTTTTDENGKYQFDNLNSGNYIVHFDKPS  780
                                                SD repeat region
781 GMTQTTTDSGDDDEQDADGEEVHVTITDHDDFSIDNGYYDDD SDSDSDSDSDSDDSDSDS 840
                                            Wall and memebrane spanning region
841 DSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSGLDNSSDKNTKDK LPDTGANED 900

901 HDSKGTLLGALFAGLGALLLGKRRKNRKNKN 931
```

Figure 4

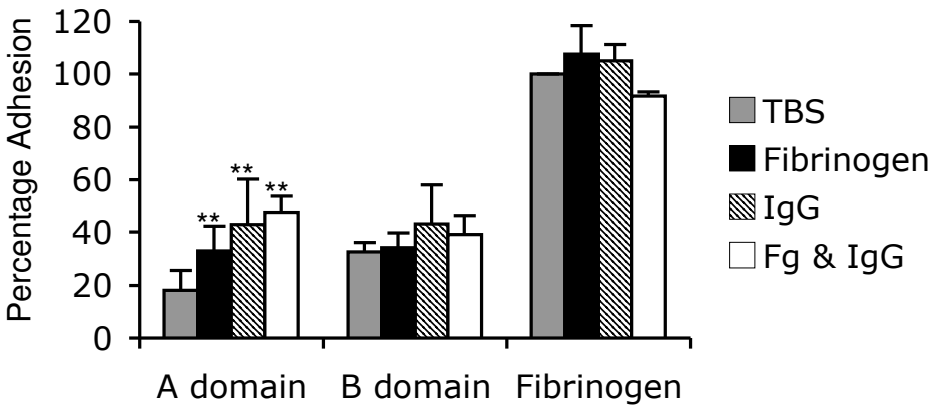

Percentage Adhesion
120
100
80
60
40
20
0
**
**
**
A domain
B domain
Fibrinogen
TBS
Fibrinogen
IgG
Fg & IgG

Figure 5

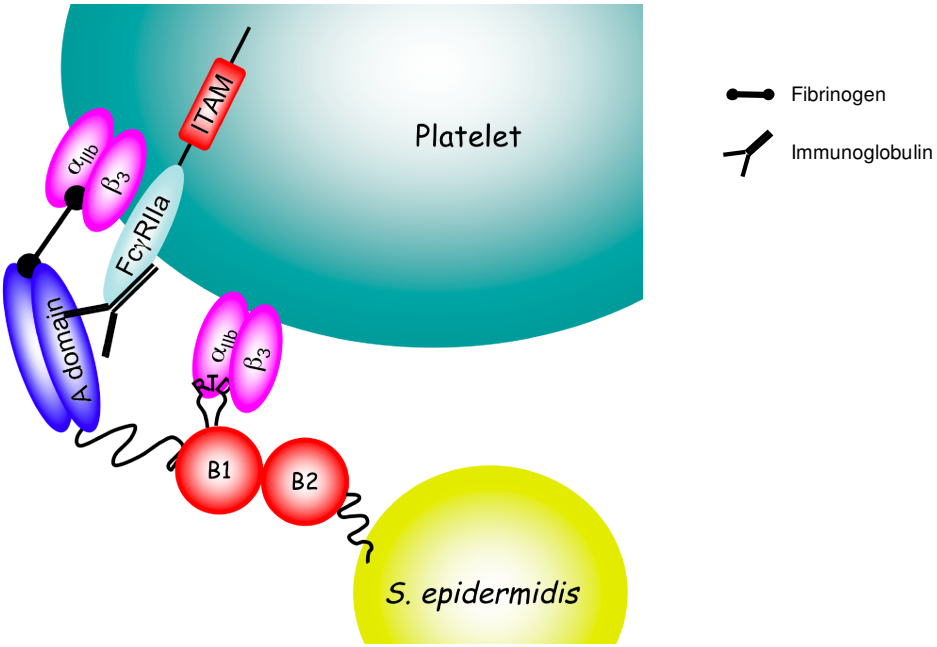

Platelet
ITAM
α_{IIb}
β_3
FcγRIIa
A domain
α_{IIb}
β_3
B1
B2
S. epidermidis
Fibrinogen
Immunoglobulin

Figure 6

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